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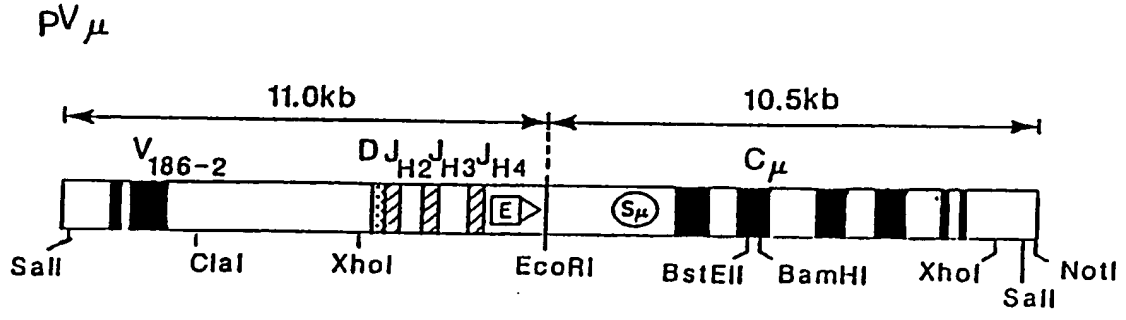

(57) Abstract

This invention provides a chimeric or transgenic non-human eukaryotic animal having incorporated into its germline unrearranged DNA fragments bearing exogenous immunoglobulin gene segments. This animal is capable of rearranging these segments and producing antibodies characterized by the presence of rearranged exogenous species Ig heavy chains.

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CHIMERIC AND TRANSGENIC ANIMALS CAPABLE OF PRODUCING HUMAN ANTIBODIES

The present invention relates generally to immunoglobulin rearrangement in chimeric and transgenic animals, and more specifically to a mouse containing in its germline, and capable of transmitting to its progeny, the ability to generate immunoglobulins characterized by the presence of human heavy chains.

Background of the Invention

Immunoglobulins are the proteins which form antibodies. The basic unit of an immunoglobulin (Ig) molecule is a complex of two identical heavy (H) and two identical light (L) polypeptide chains. Both types of chains contain a region of variable amino acid sequence at the amino terminal end (V region) in which reside the primary determinants of antigen binding specificity. Each chain also has a region of constant amino acid sequence (C region) comprising the remainder of the chain, in which reside the primary determinants of the effector functions of the antibody, such as complement fixation and antibody-dependent cellular cytotoxicity.

The variable region of heavy and light chains can be further subdivided into regions of relatively conserved amino acid sequence, framework regions, and regions of highly variable amino acid sequence, hypervariable regions or complementarity determining regions (CDRs). [See, e.g., S. Tonegawa, Nature, 302:575 (1983)].

The variable regions of both the heavy and light chain genes are assembled from component gene segments. The heavy chain variable region gene is assembled from three different elements, variable (V_H), diversity (D), and joining (J_H) segments. Families of these segments are encoded in separate clusters along the chromosome. Formation of a functional heavy chain variable region gene involves both joining of a D segment to a J_H segment and joining of a V_H segment to the pre-existing DJ_H complex to form the complete V_HDJ_H heavy chain variable region gene.

Assembly of the variable region of light chains involves the joining of a variable gene segment (V_L) to a joining segment (J_L) to form a complete V_LJ_L variable region several kilobases (kb) upstream of the DNA sequences which encode the corresponding light chain constant region (C_L). No D segments are involved in light chain variable regions.

Through the standard hybridoma technique, numerous monoclonal antibodies which are directed to a single antigenic determinant on an antigen have been developed in laboratory animals for potential human therapeutic use. To date, the majority of monoclonal antibodies used clinically have been of murine origin.

Although these reagents have been useful, a serious problem with the use of murine antibodies in humans is the neutralization of the injected antibody by host anti-Ig responses. Such host responses to mouse Ig can result in allergies, serum sickness, or immune-complex diseases. Additionally the host antibodies neutralize injected murine antibodies and rapidly clear them. The use of human immunoglobulin sequences could produce therapeutic antibodies with longer serum half-lives and possibly improved effector functions, compared to murine antibodies.

Human or partially human monoclonal antibodies have been obtained by several methods which have proven less than satisfactory for a variety of reasons. The production of mouse/human hybridomas has been limited because such lines are unstable genetically and progressively lose human chromosomes. Epstein Barr Virus transformed human B lymphocytes tend to produce small quantities of Ig and are unstable. The production of human/human hybridomas has been very limited, due to the

lack of a suitable fusion partner, the lack of sufficient numbers of human B-cells immunized against a desired antigen, poorly defined fusion protocols, and poorly defined culture conditions to support hybrid survival and antibody production. Furthermore, such cells predominantly produce antibodies of only the IgM isotype, which is not appropriate for many clinical uses. Another serious limitation with human hybridomas is the difficulty of growing such cell lines in large quantities. In vitro culture of such hybridomas is very costly and produces limited quantities of Ig compared to the large amounts that can be obtained from harvesting monoclonal antibodies from murine hybridomas.

The expression of heterologous genes from heterologous species organisms in an animal, e.g., mice, capable of expressing and regulating the foreign gene construct has been the subject of much recent research. U. S. patent 4,736,866 describes a transgenic animal carrying a recombinant activated oncogene sequence; and J. Van Brunt, Biotechnology, 6(10):1149 (October 1988). Buccini et al, Nature, 326:409 (1987) and Goodhardt et al, Proc. Natl. Acad. Sci., USA, 84:4229 (1987) refer to the rearrangement of unarranged chicken and rabbit immunoglobulin gene segments in mice. Although the genes do rearrange, this report does not show whether a diversity of variable regions can be generated at the

level of junctional diversity or combinatorial diversity, and used properly in an immune response. Additionally, a human rearranged μ transgene has been shown to mediate allelic exclusion in mice [Nussenzweig et al, Science, 236:816 (1987)].

Even more recently, human cells have been injected into immunodeficient scid mice to provide them with the capability of generating substantially human antibodies. However, each mouse capable of generating the human antibodies must be individually made. The ability to make human antibodies is not transmitted in the germline from the originally injected mouse to its progeny. Furthermore the human antibody producing cells generated in this mouse would be 'human' and therefore, presumably the same problems could arise as mentioned above in regard to production of hybridomas and EBV lines.

Thus, there remains a need in the art for an efficient and satisfactory method and compositions for providing a reliable source of human antibodies.

Summary of the Invention

As one aspect of the present invention there is provided a non-human eukaryotic animal having incorporated into its germline unrearranged DNA fragments bearing exogenous immunoglobulin (Ig) heavy chain gene

segments. The animal of the invention is capable of rearranging these segments and producing antibodies characterized by the presence of rearranged exogenous Ig heavy chains. The animal of the invention is also capable of producing an antibody also characterized by the presence of light Ig chains endogenous to said animal. Alternatively, the animal of the invention may produce antibody characterized by the presence of exogenous Ig light chains. Preferably the exogenous gene segments are of human origin.

Another aspect of this invention is an unrearranged DNA fragment for use in producing the animal of the invention. This DNA fragment is composed of at least the following elements: at least one exogenous variable Ig gene segment, at least one exogenous D Ig gene segment, at least one exogenous J Ig gene segment, and at least one μ heavy chain constant Ig region. The μ constant region is of exogenous or endogenous species origin, and is required to mediate allelic exclusion in the animal of this invention.

In a preferred embodiment, the unrearranged DNA fragment also contains an exogenous gamma constant Ig region. The unrearranged DNA fragment of the invention may also contain a switch region of exogenous or endogenous species origin, and exogenous or endogenous

species heavy chain enhancers. More preferably the exogenous segments of this unrearranged fragment are of human origin.

Still another aspect of the present invention is a method for producing the non-human eukaryotic animal of the present invention comprising introducing into the germline of said animal an unrearranged DNA fragment of this invention.

A further aspect of the present invention is a method for producing the non-human eukaryotic animal of the invention comprising transfecting into a stem cell of the animal a heterologous DNA sequence carrying unrearranged gene segments of exogenous, preferably human, Ig heavy chains. This method may permit introduction into the animal of human DNA of greater than 50kb, if desired.

Yet another aspect of the invention is a hybridoma cell line and a monoclonal antibody secreted therefrom characterized by the presence of human Ig heavy chains. The monoclonal antibody thus produced may further be characterized by the presence of either human or endogenous animal light chains. An additional aspect of the invention is a method for producing the hybridoma cell line by use of a cell of an animal of the present invention and a method for obtaining a desired monoclonal antibody of the invention.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of preferred embodiments thereof.

Brief Summary of the Drawings

Fig. 1 is a graphical illustration of a murine unrearranged DNA fragment according to the present invention; and

Fig. 2 is a graphical illustration of a more complex human unrearranged DNA fragment according to the present invention.

Detailed Description of the Invention

The present invention provides an animal developed by recombinant genetic engineering techniques which is capable of rearranging in a lymphoid specific manner introduced unrearranged exogenous, preferably human, immunoglobulin genes. The animal is thereby capable of producing in vivo resulting B cells which express and use the exogenous Ig polypeptides in a manner identical to the use of its own endogenous Igs produced in the animal's normal B cells. The animal of this invention produces in vivo a diversity of mature B lymphocytes, characterized by the presence of exogenous

Ig heavy chains. The animal is capable of using the rearranged products of the unrearranged DNA fragment, e.g., a functionally rearranged exogenous heavy chain, in an immune response. A further characteristic of the animal is an ability to transmit this ability to at least some of its progeny.

The animals of this invention are designed by the integration into their germlines of DNA carrying unrearranged or only partially rearranged exogenous Ig gene segments. The unrearranged DNA fragment carrying the gene segments may be microinjected into the fertilized egg of an animal to develop a transgenic animal or transfected into a stem cell which is used to repopulate an embryo to develop a chimeric animal. The human DNA fragments are introduced into a cell of the animal in a manner providing that the introduced DNA is stably maintained in a proportion of those cells and transmitted in the germline of the resulting animal.

To enable the animal to produce B lymphocytes characterized by exogenous Ig heavy chains, an unrearranged gene fragment of the invention is constructed to carry at a minimum DNA sequences encoding at least one exogenous variable Ig gene segment, at least one D Ig gene segment, at least one exogenous J Ig gene segment, and at least one μ constant Ig region.

Desirably the unrearranged gene fragment further contains an exogenous gamma constant Ig region.

The μ region may be of exogenous origin or from the animal itself, but is preferably human. This μ region is required to signal appropriate allelic exclusion in the resulting animal. The D gene may be of exogenous origin, e.g., human, or from the animal itself. Additionally a synthetic D gene of about 10 to 20 amino acids in length containing flanking sequences from an animal may be employed in the construct of the invention.

To further enhance the diversity of B lymphocytes produced by the animal, the unrearranged gene fragment of this invention optionally contains multiple copies of the unrearranged or partially rearranged human heavy or light chain variable and constant region gene segments.

Human gene segments for use in constructing the unrearranged DNA fragment for insertion into a cell of the selected animal of this invention include a D gene which is a short chemically synthesizable fragment. One such D gene region is described in J. V. Ravetch et al, Cell, 27:583 (1981). Other useful D regions for the unrearranged DNA fragment of the present invention are described in Y. Ichihara et al, Eur. J. Immunol., 18:649 (1988). It is expected that other D regions will eventually be isolated and their sequences determined.

These D regions may also form useful components of the unrearranged DNA fragment of the present invention in a manner analogous to the use of the above exemplary D gene segments.

However, as provided above, the D gene need not be of human origin. The flanking regions of the D gene may be of one species' origin, which the coding sequence of the D gene may be from another species. Additionally a synthetic D gene may be made by conventional techniques. Presently preferred for use in the construct are any human D or any murine D gene, a variety of which are known in the art, as identified above.

The J gene regions of the human Ig DNA sequences for use in an unrearranged DNA fragment of the present invention are published. See, e.g. Ravetch et al, cited above; and P. A. Hieter et al, J. Biol. Chem., 257:1516 (1982). The J gene region described by Ravetch, cited above, may be obtained requested from the author, based on its publication in the journal, Cell.

Additionally, μ and gamma (1 through 4) human gene sequences are published and may be purchased from the Medical Research Council (MRC) in England and employed in the unrearranged DNA fragment. See, e.g., J. W. Ellison et al, Nucl. Acids Res., 10(13):4071 (1982); N. Takahashi et al, Cell,79:671 (1982); and P. A. Hieter,

et al, Nature, 294:536 (1981). Alternatively, the constant regions may desirably be constructed by employing conventional recombinant techniques. See, e.g., T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982) and J. Sambrook et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1989). Such techniques for obtaining constant regions for use in the constructs of this invention can permit alterations deliberately designed into the recombinant genes. Such alterations or modifications of the genes can permit changed or enhanced functions, e.g., higher binding affinities, to be displayed by the gene, in comparision to isolated human genes.

Human delta, epsilon and alpha constant regions may also be employed in an unrearranged DNA fragment of this invention. These gene sequences are described in J. G. Flanagan et al, Nature,300:709 (1982). Recombinant forms of these genes are also desirably employed in the construct of this invention.

Human V gene families I through VI are also published and may be constructed by conventional recombinant techniques for insertion into the unrearranged DNA fragment of the invention. See, e.g. Berman, J. E. et al, EMBO J., 7:727 (1988).

The DNA sequences of these genes identified above may be synthesized or obtained by conventional recombinant techniques and employed in the unrearranged DNA fragment construct according to the present invention. One of skill in the art can obtain members of these gene families by employing conventional cross-hybridization techniques using a representative member of each family.

The unrearranged DNA fragment's human heavy chain gene segments, V, D, and J, will assemble with animal's own light chains during B lymphocyte maturation to generate antibodies characterized by human heavy chains. Alternatively, unrearranged human light chain gene segments may be employed in another unrearranged DNA fragment to enable the resulting animal to produce a diversity of B lymphocytes with both human heavy and light chains. The two unrearranged DNA fragments bearing the human heavy chain genes and the human light chain gene, respectively, may be injected into the same animal. Alternatively, one animal carrying the first unrearranged DNA fragment may be mated with another animal carrying the second unrearranged DNA fragment to produce progeny capable of using both unrearranged DNA fragments.

Thus an animal of this invention may be capable of generating antibodies containing human heavy chains and homologous light chains or antibodies characterized by the presence of both human heavy and light chains.

Human light chains for optional insertion in an unrearranged DNA fragment of this invention are also described in the art. See, Jirik et al, Proc. Natl. Acad. Sci., U.S.A., 83:4229 (1986) and M. Pech et al, J. Mol. Biol., 176:189 (1984).

The unrearranged DNA fragment of the invention may also contain appropriate transcriptional enhancer and promoter elements. Immediately upstream of the coding regions of the human Igs, the animal of the present invention will contain human or homologous animal, e.g., murine, constant region "switch" regions proximal to each constant region. The switch regions may also be employed in the design of the unrearranged DNA fragment which is integrated in the animal. The switch region is located immediately upstream of the coding regions of the Ig constant genes in the unrearranged DNA fragment. Such murine switch regions for use in the unrearranged DNA fragment are also published and may be sequenced or obtained by recombinant technology by one of skill in the art. See, e.g. T. Nikaido et al, Nature, 292:845 (1981) and J. V. Ravetch et al, Proc. Natl. Acad. Sci. USA,77:6734 (1980).

Similarly, Ig heavy chain enhancer sequences useful in the unrearranged DNA fragment of the invention are described in J. Banerji et al, Cell, 33:729 (1983); S. D. Gillies et al, Cell, 33:717 (1983); P. Augereau et al, EMBO J., 5(8):1791 (1986); and S. Pettersson et al, Nature, 344:165 (1990) and may be obtained by analogous methods.

Thus the constructed unrearranged DNA fragment may contain human DNA sequences as well as appropriate sequences from other non-human sources, e.g., the animal itself. In addition to the segments referenced above and described in the examples below, other human heavy and light chain gene segments or other non-human sequences, such as the switch regions, suitable promoters or enhancers, when their sequences are known, will also be useful to form unrearranged DNA fragments according to the teachings of the present invention and may be used in analogous manner to the exemplary sequences described herein. The gene segments useful in the unrearranged DNA fragments may be obtained by preparing lambda phage genomic and cDNA libraries and identifying specific Ig genes according to known techniques. See, T. Maniatis et al and Sambrook et al, cited above.

Assembly of these human Ig gene segments and other DNA segments identified above into an unrearranged DNA fragment according to the present invention employs

traditional gene assembly techniques described in T. Maniatis et al, cited above. The unrearranged DNA fragment of this invention may vary considerably in size and is preferably approximately 25 to 50 kb in length for ease of manipulation in cosmids. Larger DNA fragments of human origin may be used for construction of a chimeric animal or transgenic animal and will be propagated in yeast artificial chromosome vectors (YACS).

Specific examples of an unrearranged DNA fragment constructed of murine components, and an unrearranged DNA fragment constructed of human gene segments are disclosed, along with the procedures for constructing these fragments, in Examples 1 and 2 below.

In addition to use of the unrearranged DNA fragments described in the Examples below, animals of the invention may be produced alternatively by isolating a contiguous human DNA fragment carrying naturally unrearranged DNA sequences encoding multiple human Ig heavy chain segments, e.g., V_H, D and J_H. These naturally unrearranged fragments may be considerably larger in size than the unrearranged DNA fragment constructs described above and may require propagation in YACS vectors. An example of such a contiguous DNA fragment that could be isolated in this way is described by Berman, J. E. et al,

EMBO J., 7:727 (1988). This fragment is of 85-100 kb containing a human V gene segment, multiple human D segments, and the complete human J_H - $C\mu$ region.

Once the unrearranged DNA fragment of interest has been constructed as described above or in the examples below, or once an appropriate large human DNA fragment is isolated, these DNA sequences are propagated. The constructed unrearranged DNA fragments may be propagated in conventional plasmids or cosmids known to those of skill in the art. For example, conventional vectors useful for transgene replication are the cosmid vectors pWE15 or pWE16, supplied commercially by Stratagene, La Jolla, California. The larger chromosomal DNA sequences may be propagated in conventional yeast artificial chromosome vectors in yeast as described in D. T. Burke, et al, Science, 236:806 (1987).

Once the unrearranged DNA fragment is so propagated, the animal of the invention is developed by introducing into a cell of the animal this human DNA. This introduction is performed so that the introduced DNA is stably maintained and transmitted in the germline of the animal. Expression of functional Ig genes depends on an accurate rearrangement of DNA in the cells of the animal to bring the several gene segments e.g. variable, diversity, and joining for the heavy chain and variable

and joining for the light chain, into juxtaposition. The mechanism of allelic exclusion operates to prevent further rearrangement in a second chromosome once a complete functional unit is achieved in one chromosome.

Several advantages exist for introducing into the animal of the invention human Ig genes in an unrearranged (or partially rearranged) state in the unrearranged DNA fragment of this invention. The unrearranged or partially rearranged gene segments in the fragment allow for maximum diversity to be generated in the animal through the use of alternative V_H, D and J_H genes, and the generation of junctional diversity. Additionally the utilization of unrearranged genes is expected to lead to a more physiological response than programming an animal with completely assembled Ig genes.

Transgenic animals can be produced by several standard procedures, the most common being microinjection of the cloned unrearranged DNA fragment into one of the pronuclei of a fertilized egg according to the procedure described in R. Brinster et al, PNAS USA, 82:4438 (1985). See also, Constantini et al, J. Cell. Physiol., Suppl.:219-226 (1982); E. Robertson et al, Nature, 323:445 (1986); M. R. Kuehn et al, Nature, 326:295 (1987); M. Hooper et al, Nature, 326:292 (1987); and B. Hogan, Nature, 326:240 (1987).

The microinjected embryos are developed to term in the uterus of a pseudo pregnant animal. As many as 25% of the resulting progeny can contain the injected unrearranged DNA fragment. This procedure introduces the cloned human Ig genes into every cell of the resulting transgenic animal. Such introduced Ig genes are expressed almost exclusively in the same tissues in which the homologous endogenous genes are expressed. The animals containing the unrearranged DNA fragments generally contain the foreign DNA stably integrated in all cells. This DNA is thereafter propagated in the germline.

Alternatively, a naturally unrearranged human DNA fragment, as described above, can be introduced into isolated embryonal stem cells of the animal to create chimeric animals. Cells which have integrated the human DNA are grown in culture, and can be used to repopulate animal embryos. Such cells could be selected for those bearing the human DNA by the polymerase chain reaction, with appropriate primers [See, e.g., R. K. Saiki, et al, Science, 239:487 (1988). Alternatively a drug resistance gene, e.g., neo, may be linked to the introduced DNA and the cells selected for growth in the presence of the drug. The resulting chimeric animal contains a population of cells, some of which descend from the altered pluripotent stem cell which carry the human DNA.

Other cells of the animal descending from unaltered stem cells do not carry the human DNA. A transgenic animal can then be derived from such a chimeric animal by breeding. Such techniques for obtaining chimeric and transgenic animals are described in K. R. Thomas et al, Cell, 51:503 (1987); E. Robertson et al, Nature, 323:445 (1986); M. Hooper et al, Nature, 326:292 (1987) and M. R. Kuehn et al, Nature, 326:295 (1987).

The resulting animal of the invention is thus a chimeric or transgenic non-human eukaryotic animal having incorporated into its germline unrearranged DNA fragments bearing human immunoglobulin gene segments. The animal of the invention is capable of rearranging the human Ig gene segments to form human or partially human antibodies characterized by human heavy chains, which can be used in an immune response to an exogenous antigen.

Another characteristic of the animal of the present invention is its ability to stably maintain the human DNA fragments in its germline and to transmit the introduced DNA carrying the unrearranged heterologous human Ig gene segments to either all or a portion of its progeny. Thus, the invention provides a genetically engineered chimeric or transgenic animal capable of producing a diversity of antibodies characterized by the presence of human heavy chains.

A chimeric animal is an animal which derives a specific subset of its cells from a single particular pluripotent stem cell which is introduced into the embryo of a normal animal. That stem cell and its progeny can be distinquished from other cells of the resulting animal, for example, by containing specific DNA sequences which were present in the introduced stem cell and not in the other stem cells that eventually develop to produce the animal. Thus a chimeric animal according to this invention will have a population of cells that contain the heterologous DNA fragment containing unrearranged or partially unrearranged sequences for the heterologous Ig heavy chain genes and a population of cells which do not contain the introduced DNA. The original chimeric animal will have only a certain percentage of its progeny bearing and expressing the foreign DNA. Selective breeding of chimeric animals according to techniques known to one of skill in the art can eventually produce a transgenic animal.

A transgenic animal is an animal that carries specifically introduced heterologous DNA, such as an unrearranged DNA fragment of this invention, integrated into its genome so that the introduced sequence is present in every cell of the animal. A transgenic animal is therefore able to transmit the heterologous DNA

sequence to some or all of its progeny. If the original transgenic animal is heterozygous with respect to the foreign DNA, only some of its progeny will inherit the foreign DNA from it. If the original heterozygous transgenic animal is mated with a normal animal, the progeny will either be heterozygous or non-transgenic. If the transgenic animal is mated with another heterozygous transgenic animal, some of the progeny will be heterozygous, some will not inherit the foreign DNA at all, and some will be homozygous. The homozygous transgenic animal when mated with other homozygous transgenic animals will always produce homozygous transgenic animals carrying the foreign DNA.

According to the present invention, all animals, whether chimeric, or homozygous transgenic or heterozygous transgenic for the human DNA, will express some level of antibody characterized by the presence of human Ig heavy chains. All such animals capable of producing antibody characterized by the presence of human Ig heavy chains at any level are encompassed by the present invention.

Although any non-human eukaryotic animal may be employed in the present invention, preferably the animals will be conventionally used laboratory animals, such as rodents. More particularly, the animals of the invention are anticipated to be laboratory strains of mice.

Any animal carrying the unrearranged DNA fragment of the invention may generate a mixture of its homologous antibodies as well as antibodies characterized by human Ig heavy chains. Natural antigen-independent antibody developmental processes cause the animal to produce mature B lymphocytes to circulate in the transgenic or chimeric animal. To identify offspring carrying the human genes and/or to determine if the antibodies are characterized by human Ig heavy chains, the animal's serum or tissue may be examined using commercially available anti-human Ig antisera in an enzyme-linked immunosorbent assay (ELISA) [See, e.g., Voller A. et al, "The Enzyme Linked Immunosorbent Assay", Dynatech Europe, Borough House, Guernsey UK (1979) and Bos, E. S. et al, J. Immunoassay, 2:187 (1981)]. In addition, when the transgenic or chimeric animal is a mouse, the tail DNA is prepared and probed in a Southern blot by using a portion of the unrearranged DNA fragment as a probe. To identify appropriate rearrangement of the DNA fragment in the resulting animal, the spleen of the animal is removed by conventional splenectomy (spleen cells contain predominantly B lymphocytes) and the tissue examined on a Southern blot for hybridization and rearrangement. An alternative method of detecting appropriate Ig rearrangement is to prepare RNA from central (fetal liver and bone marrow) and peripheral

(spleen) lymphoid organs and assay for expression of the rearranged DNA in the fragment via Northern blot analyses with probes specific for human V_H and C regions.

To obtain animals containing larger numbers of human V_H genes than the numbers present on each individual unrearranged DNA fragment construct, strains of transgenic or chimeric animals containing different human Ig genes are mated, by standard breeding techniques, and progeny selected that contain multiple different human Ig transgenes. Such progeny are identified by the procedures described above.

Hybridoma cell lines secreting monoclonal antibodies directed to a particular antigenic epitope may be developed from the chimeric or transgenic animals of the present invention. To obtain such hybridoma cell lines, the animal is immunized with a selected antigen, for example, a tumor antigen (to generate an anti-tumor antibody), an antibody to an infectious agent, e.g., a bacterium or virus, e.g., HIV, or other antigenic substance of choice.

The mature B lymphocytes in the animals of the invention then generate a specific antibody response to the antigen. Such an antibody response would include heavy chains expressed from the foreign DNA fragments, now rearranged. Once the unrearranged DNA fragments in the animals have functionally rearranged to produce a

human Ig heavy chain and/or Ig light chain, during the pre-B cell stage, the remainder of the differentiation of the B cells is normal. Spleen B cells are isolated from the animals after primary or secondary immunization.

Traditional Kohler and Milstein techniques are employed and the splenic B lymphocytes fused with a selected myeloma cell line. This fusion results in a hybridoma cell line, which when cultured in appropriate culture medium and under suitable conditions known to those of skill in the art secretes a selected monoclonal antibody. The development of a hybridoma cell line according to this invention is not limited to the selection of specific myeloma cell lines or antigens.

These hybridoma cell lines are screened by standard techniques for the production of anti-specific antigen antibodies. Hybridomas which use the transgenic heavy chain genes to produce antibody are identifiable by use of a transgene specific probe. Exemplary probes are antisera against the human Ig constant regions.

The transgene construct may also incorporate a DHFR or other selectable and/or amplifiable marker. This marker gene may be linked with an independent promoter element, such as DHFR cDNA linked to an SV40 promoter and polyadenylation sequences. Alternatively, DHRF cDNA may be placed immediately downstream of an encephalomyelocarditis virus or polio virus leader

sequence without an independent promoter element [See, e.g., J. Pelletier et al, Nature, 334:320 (1988); S. K. Jang et al, J. Virol., 63:1651 (1989)].

This latter structure may be placed immediately downstream of the coding sequence of the secreted exon terminus for the heavy chain constant region, upstream of necessary polyA addition sequences. This ensures the presence and expression of the DHFR message that is expected in resulting hybridomas expressing the Ig transgene. If the structure is attached in this manner to the gamma constant region in a construct similar to that of Example 2 with μ and gamma constant regions, selection for expresion of DHFR by conventional means would allow selection of cells that have class switched from transgene μ to gamma expression. In either strategy, the presence of the marker gene would allow selection for the maintenance of the chromosome bearing the transgene and may amplify transgene sequences.

These monoclonal antibodies characterized by the presence of human Ig heavy chains have great potential therapeutic use in the treatment of a variety of human disorders amenable to antibody treatment. The presence of the human heavy chains in these antibodies are likely to be considerably less provocative of an anaphylactic response in a human patient than antibodies characterized by the presence of non-human heavy chains.

Monoclonal antibodies have immense potential for use as prophylactic, diagnostic, and therapeutic agents in the treatment of various types of human ailments. Potential or actual applications of these reagents include the provision of passive immunity against various microorganisms and viruses when injected into human patients. Also, these antibodies may be employed in the neutralization of viruses, parasites, and bacteria, for example, bacterial toxins or snake venoms.

The antibodies may also be used to neutralize pharmaceutical drugs with low toxicity thresholds, such as digoxin, or in treating drug overdoses. Additionally such antibodies may neutralize B lineage cells producing autoimmune antibodies or T lineage cells producing cell-mediated self-destructive responses. Among other conditions which can be neutralized by use of these antibodies are allergic responses. Significantly, the neutralization of tumor cells is another important area of therapy for the antibodies of this invention. These antibodies may also be employed in mediating normal antibody functions.

Other methods for use of these antibodies are in antibody dependent cell mediated cytotoxicity, or as a targeting agent for another therapeutic reagent or a detectable label for diagnosis, e.g., a radioactive label.

The following examples illustrate the construction of unrearranged DNA fragments according to the invention and the development of chimeric and transgenic animals carrying these fragments. These examples do not limit the invention and are descriptive only.

Example 1 - Construction of an Unrearranged Murine Immunoglobulin Gene in a Cosmid Vector

An unrearranged DNA fragment construct according to this invention is called pVμ [See Fig. 1A]. It contains the murine germline V_{186-2} segment [Bothwell, A.L.M. et al, Cell, 24:625 (1981)], which is known to be used in the antibody response to 4-hydroxy-3-nitrophenyl (NP). A panel of hybridomas are generated from the transgenic mice containing this construct, immunized with the NP-hapten conjugated to keyhole limpet hemocyanin.

This V segment is upstream of a D_{Q52}-J_H2 rearrangement, with unrearranged J_H3 and J_H4 segments and the heavy chain enhancer downstream. This D_{Q52}-J_H2 rearrangement is generated by recombination of these sequences after transfection into the Abelson murine leukemia virus-transformed pre-B cell line, 38B9 [Blackwell, T.K. and Alt, F. W., Cell, 37:105 (1984)].

The nucleotide sequence of this DJ_H join, called I12-2, has been determined [Blackwell, T. K. et al, Nature, 324:585 (1986]. The "spacer" between the 3' end of the V_{186-2} coding region and the $D_{JH}2$ segment is comprised of approximately 3.5 kb of sequence derived from the V_{186-2} genomic clone and 3 kb of the sequence upstream of the genomic D_{Q52} segment. Downstream of the heavy chain enhancer is the coding sequences for the murine μ constant region, derived from a genomic clone, called pμ5 [Alt, F. W. et al, Cell, 27:381 (1981)]. This clone contains an approximately 10.5 kb Eco RI fragment bearing the μ coding region. The entire Eco RI fragment is incorporated into pVμ.

This construct is designed with the intention of excising the V_{186-2}-DJ_H-μ region on a single Not I fragment of approximately 22 kb, that can be purified for microinjection. Such a DNA fragment construct contains no vector DNA that could potentially interfere with the rearrangement or expression of the immunoglobulin gene.

The cosmid construct, pVμ is prepared as follows:

i) The 10.5 kb genomic μ fragment is cloned into a derivative of pSP65, called pSP65N, having a unique Not I site. Another plasmid for this use is the Bluescript plasmid [Stratagene, La Jolla, CA]. Plasmid pSP65N is prepared from pSP65 [Promega Biotec, Madison,

WI] by destroying the unique HindIII site, and replacing it with a unique Not I site using synthetic oligodeoxy ribonucleotides. The genomic μ fragment is an Eco RI fragment, and therefore, can be cloned into Eco RI-digested pSP65N. Using appropriate restriction enzymes that cleave within the μ sequence, such as Bgl II and Xho I, a clone can be selected that has the 3' end of the fragment. This plasmid is called pSP65Nμ.

ii) The V_{186-2} and $D_{JH}2$ segments are on Eco RI fragments of approximately 7 kb and 4 kb, respectively. The Eco RI fragment bearing V_{186-2} is cloned into the Eco RI site of pSP65N, and a clone selected that has the Not I site of the plasmid at the 5' end of the V_{186-2} coding region. The Eco RI site proximal to the Not I site is destroyed, by carrying out a partial Eco RI digestion, purifying the single cut DNA from a low melt agarose gel, digesting with T4 DNA polymerase in the presence of all of the four different deoxynucleoside triphosphates, and religating. After bacterial transformation with the ligation products, a clone is identified by restriction enzyme digest analysis with only the Eco RI site proximal to the Not I site destroyed. This clone is called pSP65NV.

iii) pSP65NV is digested with Eco RI, and the Eco RI fragment bearing the DJ_H2 segment is ligated into this site. A resultant clone is selected that has the V

and DJ_H2 segments in the correct orientation. Such a clone has a Xho I site immediately upstream of the DJ_H2, and 2.4 kb from the 3' Eco RI site. The internal Eco RI site of this clone is destroyed, by a similar procedure to that used in (ii) above. The resultant clone, called pSP65NVDJ, has a Not I-Eco RI fragment of approximately 11 kb.

iv) pVμ is constructed by ligating the 11 kb Not I-Eco RI fragment of pSP65NVDJ with the 10.5 kb Eco RI-Not I μ fragment generated by complete Not I - partial Eco RI digest of pSP65Nμ, in the presence of a Not I - digested cosmid vector. A derivative of the cosmid vector pWE15 [Stratagene, La Jolla] is used in the construction of this unrearranged DNA fragment construct in which the SV40 promoter-neo gene cassette has been replaced by a mutant dihydrofolate reductase (DHFR) gene. This gene is useful as a dominant selectable marker. This gene is incorporated into the DNA construct flanking the Ig gene segments, and is used for co-amplifying and increasing the expression of the rearranged DNA in resultant hybridomas from the transgenic animal. An example of the use of a mutant DHFR gene to amplify the expression of linked DNA sequences is provided by H. Dora et al, J. Immunol., 139:4232 (1987).

This ligation is carried out under standard conditions for cosmid ligations. The ligation products are incubated with in vitro bacteriophage lambda packaging extracts, and then used to infect E. coli HB101. The resultant colonies are screened by restriction enzyme digestion analysis, and in situ hybridization to the different fragments that make up the desired construct.

Example 2 - Construction of an Unrearranged Human Ig Gene in a Cosmid Vector

Another unrearranged DNA fragment construct according to this invention comprises an unrearranged human V_H gene segment, the human J_H locus with a single upstream, unrearranged D segment, the murine μ gene including its upstream μ switch region, the murine gamma 2b switch region, and the human gamma 1 coding region. The murine μ may be changed for the human μ region, since both regions have been found to signal allelic exclusion in transgenic mouse models. Human switch regions may also be substituted for switch regions of murine origin.

i) The human 1-9II V_H gene segment of the V_H4 family described in Berman, J. E. et al, EMBO J., 7:727 (1988) present on an Eco RI fragment of approximately 2.4 kb, is cloned into Eco RI-digested pSP65N. A recombinant clone is selected that has the vector Not I site at the

5' end of the V_H sequence. The Eco RI site adjacent to the Not I site is destroyed, as described in Example 1. Alternatively the 8-1B V gene segment of the V_H3 family could be excised on an EcoRI fragment, and used in the same manner. The resulting clone is digested with Eco RI, and the human J_H fragment is cloned into it. This J_H fragment also includes one unrearranged D segment [J. V. Ravetch et al, Cell, 27:583 (1981)].

A clone, called pSP65NHVJ, is selected in which the J_H locus is in the correct orientation to the V_H segment, placing the D segment between the V_H and J_H segments.

ii) The murine germline μ gene found on plasmid pμ [Grosschedl et al, Cell, 38:647 (1984)], is excised therefrom on an approximately 10.5 kb Eco RI-Xho I fragment. This fragment is cloned into the Bluescript plasmid digested with Eco RI and Xho I. The resulting clone is then digested with Xho I, which cleaves in the vector polylinker. The cleaved DNA is treated with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates, and then ligated with synthetic DNA linkers containing an Sfi I site. The DNA is then digested with Sfi I and religated. The resulting plasmid, pμSfi I contains an approximately 10.5 kb genomic fragment, with a unique Eco RI site at the 5' end, and a unique Sfi I site at the 3' end.

iii) The murine gamma 2b switch region is excised from the genomic clone SL1 in a bacteriophage lambda vector, on an Eco RI-HindIII fragment of approximately 4.4 kb [See, e.g., Stanton and Marcu, Nucl. Acids Res., 10:5993 (1982)]. This DNA is cloned into the Eco RI and HindIII sites of the Bluescript plasmid. The 5' vector Not I site is then destroyed, and replaced with the same synthetic Sfi I linkers, as described in (ii). Then the 3' vector Sal I site is destroyed, and synthetic Not I linkers are used to create a new Not I site. The resulting clone is called pBS gamma 2b.

iv) The human gamma 1 gene is excised from the clone HG3A on a HindIII fragment [Ellison, J. W. et al, Nucleic Acids Res., 10:4071 (1982)]. This HindIII fragment is cloned into the HindIII site of pBS gamma 2b. A recombinant clone, called pBS gamma 1, is selected that has the gamma 1 gene in the correct orientation, with respect to the gamma 2b switch region. The same or similar gamma 1 fragments may be obtained from other genomic human gamma 1 clones, if desired.

v) The following fragments are then prepared: (1) a Not I-partial Eco RI V_H-D-J_H fragment of pSP65NHVJ (2 kb); (2) an Eco RI-Sfi I μ fragment of pμSfi I (10.5 kb); and (3) a Sfi I-Not I Sgamma 2b-human gamma 1 fragment of pBS gamma 1.

These fragments are then ligated with a Not I digested cosmid vector, under standard cosmid ligation conditions, as in Example 1. The ligation products are packaged, and used to transform E. coli HB101. Clones are identified that contain a V-D-J_H-μ-S gamma2b-gamma 1 unrearranged DNA fragment construct. Such a construct is excised from the clone by Not I digestion, and purified for microinjection.

Example 3 - Production of a Transgenic Mouse

The unrearranged DNA fragment constructs described in Examples 1 and 2 are employed to produce transgenic mice, as follows:

Transgenic mice were obtained essentially as described by B. Hogan et al, "Manipulating the Mouse Embryo, A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1986) using mouse F_1 strain C57BL6 X CBA. Thirty-six offspring are obtained from a series of injections of the DNA construct described in Example 1. Tail DNA preparations from these mice are digested with Eco RI and Xho I, and examined by Southern blot hybridization using a J_H locus probe. All mice are found to have the germ line J_H locus bands of 2.9kb and 3.5kb. In addition, ten mice are found to have a 2.4kb band, which is the size predicted for the XhoI-EcoRI fragment containing the DJ_H2 segment of the

unrearranged DNA fragment. Thus, these ten mice are transgenic, according to the invention.

To determine whether or not V to DJ rearrangement is occurring in the transgene in the B cells of the transgenic mice, survival splenectomy is performed on two of the founder transgenic mice. Preliminary Southern blot analysis indicates that they contain multiple copies of the unrearranged DNA construct. The spleen DNA prepared from the mouse designated 1763 is digested with SalI and EcoRI and examined by Southern blot hybridization to a J_H locus probe. SalI cuts at the 5' end of the construct, and EcoRI cuts downstream of J_H.

SalI-EcoRI double digestion of the unrearranged DNA fragment construct should generate an 11kb fragment containing V186-2 and DJ_H2. Rearrangement of these two gene segments and deletion of the spacer DNA is predicted to generate a SalI-EcoRI fragment of approximately 4kb. By analysis of this Southern blot, tail DNA of 1763 is found to have an unrearranged band of 11kb, and the endogenous J_H band of approximately 6.2kb. In spleen DNA, in addition to 11 and 6.2kb bands, a 4kb band is found, indicating rearrangement of the DNA fragment in the spleen but not the tail tissue of the animal.

To further characterize this DNA construct rearrangement, the remaining spleen DNA preparation of mouse 1763 is digested with SalI and EcoRI, and the DNA in the 3.5-4.5kb size range is eluted from a preparative agarose gel. This DNA is cloned into the bacteriophage lambda vector, lambda ZAP [Stratagene]. The resulting plaques are screened with a J_H probe and picked. The inserts are converted to plasmids using the lambda ZAP excision method. These plasmids are rescreened with the same probe, and positive colonies isolated. The nucleotide sequence of each insert is then determined using synthetic oligonucleotides to prime chain synthesis. Two oligonucleotides are employed, one hybridizing to the 3' end of J_H2, and the other hybridizing to the 5' end of V_{186-2}.

The nucleotide sequence of four of the DNA fragment rearrangements from spleen DNA are determined from the ATG initiation codon of V_{186-2} to the 3' end of the DJ_H2 segment. From the sequence of the V_H-to-DJ_H joins of the first four rearranged V region sequences, the DNA fragments are observed to be rearranged with junctional variation occurring. These rearranged V_H-DJ_H joins appear indistinguishable from those expected for endogenous gene segments.

In two of the four sequences, there is a loss of one or more base pairs from the 3' end of V_{186-2}. In all four sequences, there is a loss of two or more base-pairs from the 5' sequence of the DJ_H segment. In three of the four sequences, "N-regions" are inserted between the recombined V and DJ_H segments of one or three base-pairs. Although this sample of junction sequences is small, there appears to be a preference for G-C base-pairs in the N-regions. This observation is consistent with the known nucleotide preference of terminal deoxynucleotidyl transferase, which has been implicated in adding the extra bases of the N-region to such junctions. [See, e.g., F. W. Alt et al, Proc. Natl. Acad. Sci. USA, 79:4118 (1982) and Kurosawa et al, J. Exp. Med., 155:201 (1982)].

The sequences of the rearranged DNA fragment V regions are identical, outside the region of the V_H-to DJ_H join, and identical to the known sequences of V_{186-2} and J_H2. Thus, no somatic mutation occurs in the sequenced rearranged DNA constructs, a result that would be expected from the analysis of rearrangements in total spleen DNA.

Numerous modifications and variations in practice of this invention are expected to occur to those skilled in the art. For example, as more human immunoglobulin gene sequences are identified, one of skill in the art can easily apply the teaching of the this invention to construct appropriate transgenes for the preparation of a transgenic animal capable of expressing an antibody characterized by the presence of that particular immunoglobulin. Such modifications therefore are believed to fall within the scope of this invention.

CLAIMS

1. A non-human eukaryotic animal having incorporated into its germline unrearranged DNA fragments bearing exogenous immunoglobulin gene segments, said animal capable of rearranging said segments and producing antibodies characterized by the presence of rearranged exogenous species Ig heavy chains.

2. The animal according to claim 1 wherein said exogenous segments are human immunoglobulin gene segments.

3. The animal according to claim 1 selected from the groups consisting of a chimeric animal, a heterozygous transgenic animal or a homozygous transgenic animal.

4. The animal according to claim 2 comprising a rodent.

5. The animal according to claim 4 comprising a mouse.

6. The animal according to claim 1 capable of producing said antibody characterized by the presence of light Ig chains endogenous to said animal.

7. The animal according to claim 1 capable of producing said antibody characterized by the presence of exogenous species immunoglobulin light chains.

8. The animal according to claim 7 wherein said light chains are of human origin.

9. The animal according to claim 1 wherein said gene segments comprise at least one exogenous variable immunoglobulin gene segment, at least one D immunoglobulin gene segment, at least one exogenous J immunoglobulin gene segment, and at least one μ heavy chain constant immunoglobulin region.

10. The animal according to claim 2 wherein said gene segments comprise at least one human variable immunoglobulin gene segment, at least one D immunoglobulin gene segment, at least one human J immunoglobulin gene segment, and at least one μ heavy chain constant immunoglobulin region.

11. The animal according to claim 10 wherein said μ constant region is of human or murine origin.

12. The animal according to claim 9 wherein said gene segments further comprise an exogenous gamma heavy chain constant region.

13. The animal according to claim 10 wherein said gene segments further comprise a human gamma heavy chain constant region.

14. An unrearranged DNA fragment for use in producing the animal of claim 1 comprising substantially the construct of FIG. 1.

15. An unrearrangement DNA fragment for use in producing the animal of claim 2 comprising substantially the construct of FIG. 2.

16. An unrearranged DNA fragment for use in producing the animal of claim 1 comprising substantially at least one exogenous variable immunoglobulin gene segment, at least one D immunoglobulin gene segment, at least one exogenous J immunoglobulin gene segment, and at least one μ constant immunoglobulin region.

17. An unrearranged DNA fragment for use in producing the animal of claim 2 comprising substantially at least one human variable immunoglobulin gene segment, at least one D immunoglobulin gene segment, at least one human J immunoglobulin gene segment, and at least one μ constant immunoglobulin region.

18. The fragment according to claim 17 wherein said μ constant region is of human or murine origin.

19. The fragment according to claim 16 wherein said gene segments further comprise an exogenous gamma constant region.

20. The fragment according to claim 17 wherein said gene segments further comprise a human gamma constant region.

21. The fragment according to claim 16 comprising a switch region.

22. The transgene according to claim 16 comprising an immunoglobulin heavy chain enhancer.

23. The fragment according to claim 17 comprising a murine switch region.

24. The fragment according to claim 17 comprising an immunoglobulin heavy chain enhancer.

25. A method for producing a non-human eukaryotic animal having incorporated into its germline unrearranged DNA fragments bearing exogenous immunoglobulin gene segments, said animal capable of rearranging said segments and producing antibodies characterized by the presence of rearranged exogenous Ig heavy chains comprising microinjecting into a cell of said animal the fragment of claim 16.

26. A method for producing a non-human eukaryotic animal having incorporated into its germline unrearranged DNA fragments bearing human immunoglobulin gene segments, said animal capable of rearranging said segments and producing antibodies characterized by the presence of rearranged human Ig heavy chains comprising microinjecting into a cell of said animal the fragment of claim 17.

27. A hybridoma cell line secreting a monoclonal antibody characterized by the presence of human immunoglobulin heavy chains.

28. A monoclonal antibody characterized by the presence of human immunoglobulin heavy chains produced by an animal according to claim 2.

29. A method for producing a hybridoma cell line secreting a monoclonal antibody characterized by the presence of human immunoglobulin heavy chains comprising introducing into the animal of claim 1 a selected antigen, and fusing splenic B cells from said animal with a selected myeloma cell or plasmacytoma.

30. A method for producing a monoclonal antibody characterized by the presence of human immunoglobulin heavy chains comprising culturing the cell line of claim 29 in suitable medium and collecting the antibody secreted therefrom.

31. A method for producing a non-human eukaryotic animal having incorporated into its germline unrearranged DNA fragments bearing human immunoglobulin gene segments, said animal capable of rearranging said segments and producing antibodies characterized by the presence of rearranged human Ig heavy chains comprising transfecting into a stem cell of said animal a human DNA sequence carrying unrearranged gene segments of human immunoglobulin heavy chains.

FIG. 1

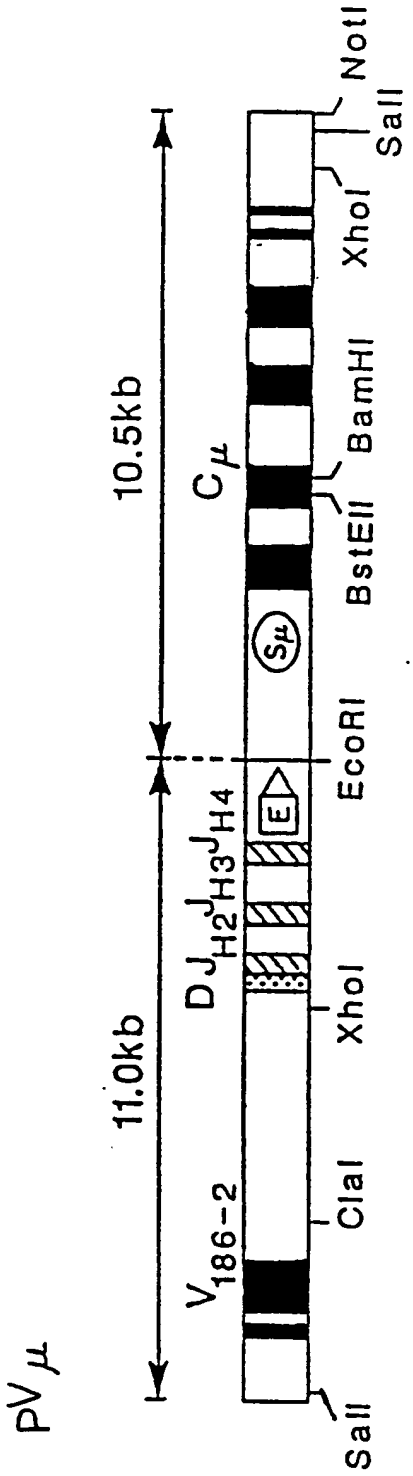
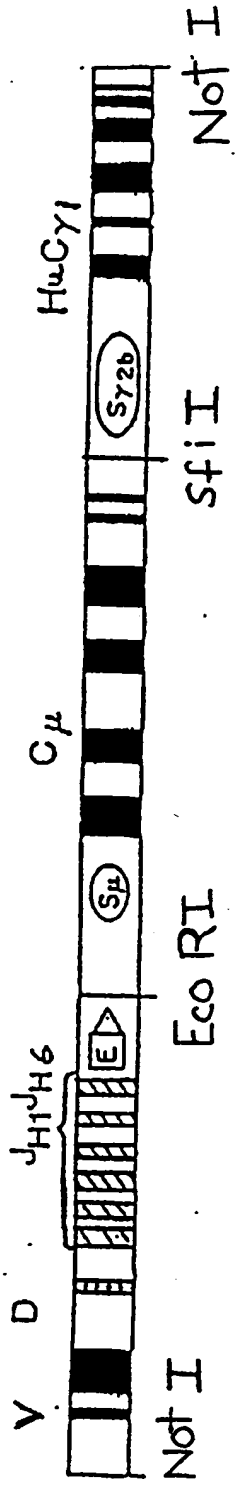

NotI
SalI
XhoI
BamHI
BstEII
EcoRI
XhoI
ClaI
SalI
10.5kb
11.0kb
Cμ
Sμ
E
DJH2JH3JH4
V186-2
PVμ

FIG. 2



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/03894

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/00 ; C12N5/20 ; C12P21/08 ; C12N15/06

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, no. 7, September 1989, WASHINGTON US pages 6709 - 6713; BRUGGEMANN, M. et al.: "A repertoire of monoclonal antibodies with human heavy chains from transgenic mice" see the whole document ---	1-6, 9-11, 14, 16-18, 25-31

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 NOVEMBER 1990	07. 12. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

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